SEPARATION OF UNKNOWN SAMPLE USING TLC TECHNIQUE

1. Introduction

1.1. Separation technique definition

In chemistry, a separation process, or a separation technique, or simply a separation, is a method to achieve any mass transfer phenomenon that converts a mixture of substances into two or more distinct product mixtures (which may be referred to as fractions). In some cases, a separation may fully divide the mixture into its pure constituents. Separations are carried out based on differences in chemical properties or physical properties such as size, shape, mass, density, or chemical affinity, between the constituents of a mixture.

1.2. The purpose of a separation

The purpose of a separation may be analytical, i.e. to help analyze components in the original mixture without any attempt to save the fractions, or may be preparative, i.e. to "prepare" fractions or samples of the components that can be saved. The separation can be done on a small scale, effectively a laboratory scale for analytical or preparative purposes, or on a large scale, effectively an industrial scale for preparative purposes, or on some intermediate scale.

1.3. Classifying separation techniques

Mixtures come in many forms and phases. Most of them can be separated, if there is a significant difference in at least one of their chemical or physical properties. Table 1 provides a partial list of separation techniques, classified by the chemical or physical property being exploited.

Table 1: Classification of Separation Techniques

Basis of Separation	Separation Technique
size	Filtration, Dialysis, Size-exclusion chromatography
mass or density	Centrifugation
complex formation	masking
change in physical state	Distillation, Sublimation, Recrystallization
change in chemical state	Precipitation, electro-deposition, Volatilization
partitioning between phases	Extraction, chromatography

The separation techniques that are routinely used including the following:

1.3.1 Filtration separation method

This is a more common method of separating an insoluble solid from a liquid. An example of such a mixture is sand and water. Filtration is used in water treatment plants, where water from the river is filtered to remove solid particles. This process involves the use of a **filter paper** placed in a filter funnel. The funnel is placed in a beaker and the mixture of water and sand is poured into the funnel. The liquid part drains through the filter paper into the beaker, leaving the solid sand particles trapped on the filter. In filtration, the liquid part collected is called the **filtrate** and the solid bit that remained on the filter paper is called the **residue**.

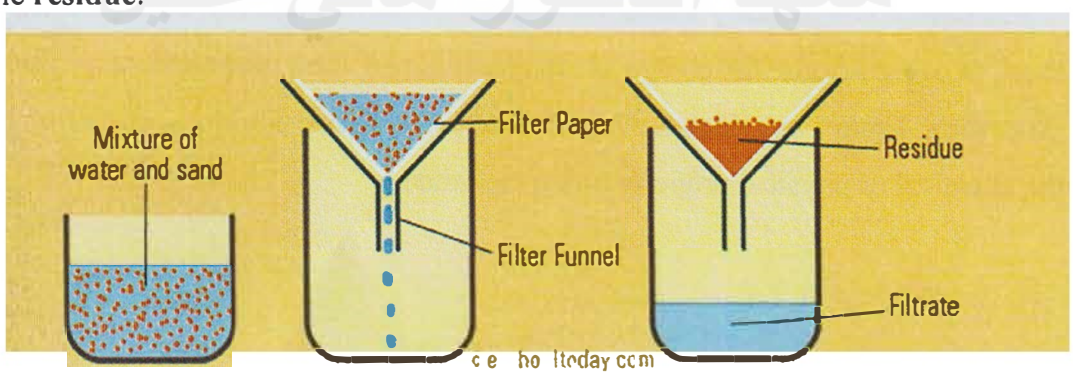


Figure 1: scheme represents a basic lab setup for filtration

1.3.2 Simple distillation

This method is best for separating a liquid from a solution. In a way, the concept is similar to evaporation, but in this case, the vapor is collected by **condensation**. For example, if you want to separate water from a salt solution, simple distillation would be great for this. A beaker of the salt solution is heated to the boiling point of the liquid. As it boils, the liquid turns into vapor (gas). The vapor is directed through tubes (condenser) connected to another beaker. As the vapor goes through the tube, it is cooled down by running cold water around the tubes. This forces the temperature of the vapor to fall, causing the gas to turn into liquid again (condensation). The liquid is pure at this point, as it is free from salt. The process continues until all the liquid in the solution turns into vapor, leaving the salt residue. The distilled liquid is called a 'Distillate'

Similar to simple distillation; fractional distillation is best for separating a solution of two **miscible liquids**. (Miscible liquids are liquids that dissolve in each other). The Fractional method takes advantage of the **different boiling points** of the two liquids.

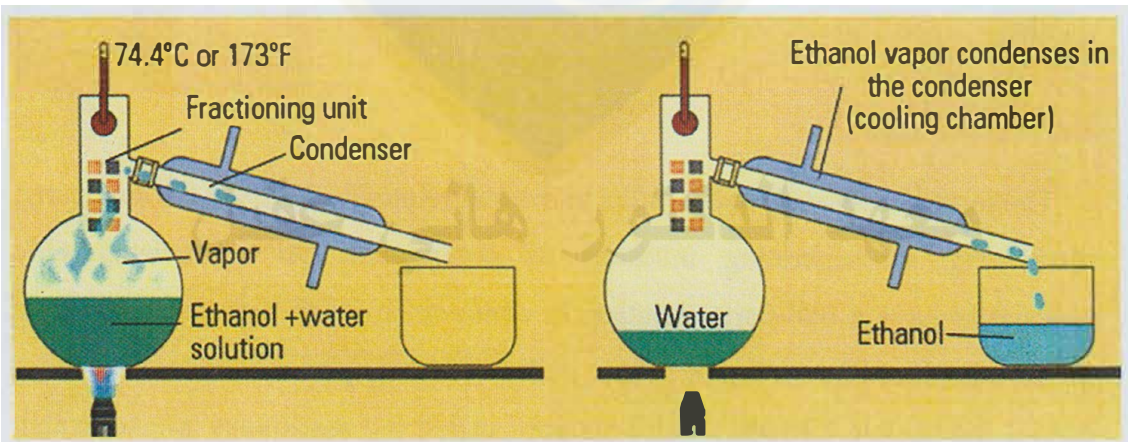


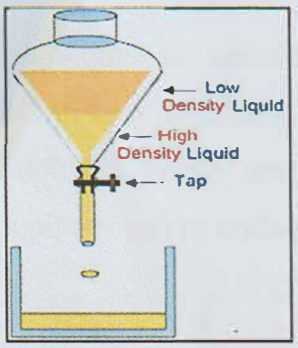
Figure 2: The separation of ethanol from water by fractional distillation

1.3.3. Funnel separation

In this technique, two liquids that do not dissolve very well in each other (immiscible liquids) can be separated with the concept of unequal density. A mixture of oil and water, for example, can be separated by this technique. The two liquids in the mixture have different densities, making the heavier liquid settle at the base of the container.

1.3.4. Centrifugation

Sometimes the solid particles in a liquid are very small and can pass through a filter paper. For such particles, the filtration technique cannot be used for separation. Such mixtures are separated by centrifugation. So, **centrifugation is the process of separation of insoluble materials from a liquid where normal filtration does not work well.** The centrifugation is based on the size, shape, and density of the particles, viscosity of the medium, and the speed of rotation. The principle is that the denser particles are forced to the bottom and the lighter particles stay at the top when spun rapidly.



1.4. Chromatography

The term **chromatography** refers to several related techniques for analyzing, identifying, or separating mixtures of compounds. All chromatographic techniques have a two-part operation in common. In each technique a sample mixture is placed into a liquid or gas, called a **mobile phase**. The mobile phase carries the sample through a solid support, called the **stationary phase**, which contains an adsorbent or another liquid. The different compounds in the sample mixture move through the stationary phase at different rates, due to different attractions for the mobile and stationary phases. Thus, individual compounds in

the mixture separate as they move through the stationary phase. The separated compounds can be collected or detected, depending on the particular chromatographic technique involved.

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for more advanced use (and is thus a form of **purification**). Analytical chromatography is done normally with smaller amounts of material and is usually used for identification.

1.4.1. Types of chromatography

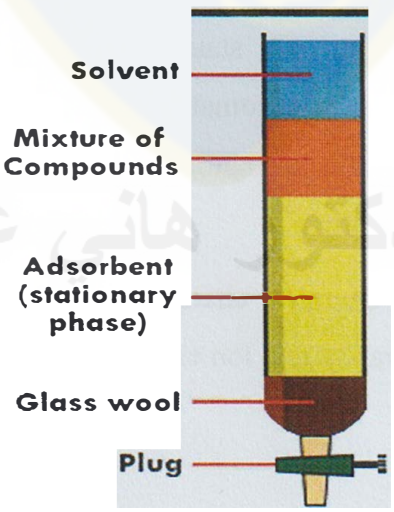
There are different types of chromatography. The differentiation is based on techniques of chromatography or principle or physical changes used.

- The physical states of stationary phase and mobile phases (homogenous or heterogonous techniques).
- The on principle of separation used (including adsorption and partition chromatography).
- The chemical nature of stationary phase and mobile phases used (polarity).
- Based on the shape of stationary phase employed (column chromatography and planar chromatography).
- Based on purpose of chromatography experiment (preparative or analytical chromatography).
- Based on physical or chemical character of the stationary phase (e.g size exclusion chromatography and ion exchange chromatography.

1.4.1.1 Column chromatography

Column chromatography is a separation technique in which the stationary bed is within a tube. The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open. Differences in rates of movement through the medium are calculated to different retention times of the sample.

In 1978, W. Clark Still introduced a modified version of column chromatography called **flash column chromatography**. The technique is very similar to the traditional column chromatography, except for that the solvent is driven through the column by applying positive pressure. This allowed most separations to be performed in less than 20 minutes, with improved separations compared to the old method. Modern flash chromatography systems are sold as pre-packed plastic cartridges, and the solvent is pumped through the cartridge. Systems may also be linked with detectors and fraction collectors providing automation. The introduction of gradient pumps resulted in quicker separations and less solvent usage.

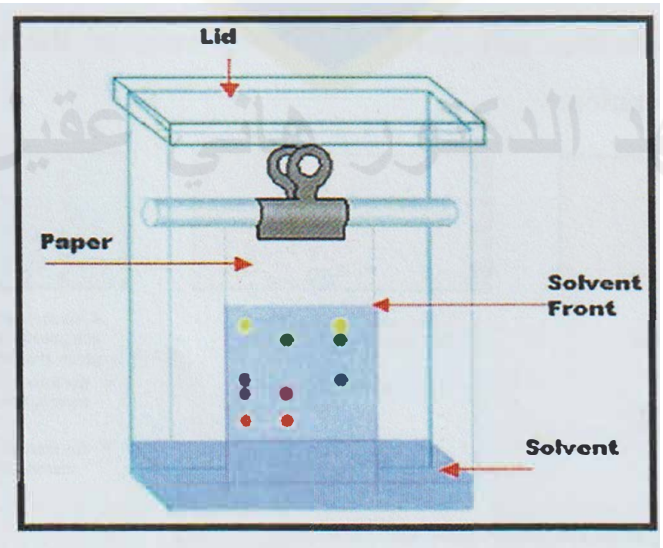


1.4.1.2 Planar chromatography

Is a separation techniquc in which the stationary phase is present as or on a plane. The plane can be a paper, serving as such or impregnated by a substance as the stationary bed ***(paper chromatography)*** or a layer of solid particles spread on a support such as a glass plate ***(thin layer chromatography).*** Different compounds in the sample mixture travel different distances according to how strongly they interact with the stationary phase as compared to the mobile phase. The specific Retention factor (R_f) of each chemical can be used to aid in the identification of an unknown substance.

1.4.1.3 Paper chromatography

Is a technique that involves placing a small dot or line of sample solution onto a strip of *chromatography paper*. The paper is placed in a container with a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture, which starts to travel up the paper with the solvent. This paper is made of cellulose, a polar substance, and the compounds within the mixture travel farther if they are non-polar. More polar substances bond with the cellulose paper more quickly through H-bond, and therefore do not travel as far.



1.5. Thin layer chromatography (TLC)

It is a **simple** and **inexpensive** analytical technique that can **quickly** and **efficiently** separate quantities of less than ten micrograms of material. TLC has many applications in the organic laboratory. TLC is used for the rapid analysis of reagent and product purity, or to quickly determine the number of compounds in a mixture. Also, by comparing an unknown compound's behavior to the behaviors of known standard compounds, mixture compounds can be tentatively identified.

1.5.1 Principle of TLC

The technique is based on a polarity interplay between the sample and two other substances called the **solid (or stationary) phase** which is finely ground silica or alumina particles coated on plastic or aluminum sheet or glass slide to produce TLC plate , and the **mobile phase**, which is a liquid called the **eluting solvent**. The sample to be separated first becomes **adsorbed** onto the surface of the solid phase through polar interactions. More polar samples adhere (or bind) more strongly than less polar ones. The mobile phase then comes in and sweeps across the stationary phase, competing for the sample. The more polar the solvent, the greater its capacity to carry the components of the mixture with it, "yanking" them away from the solid phase as it moves. Different components with different polarities will travel at different rates as the solvent moves, causing their separation.

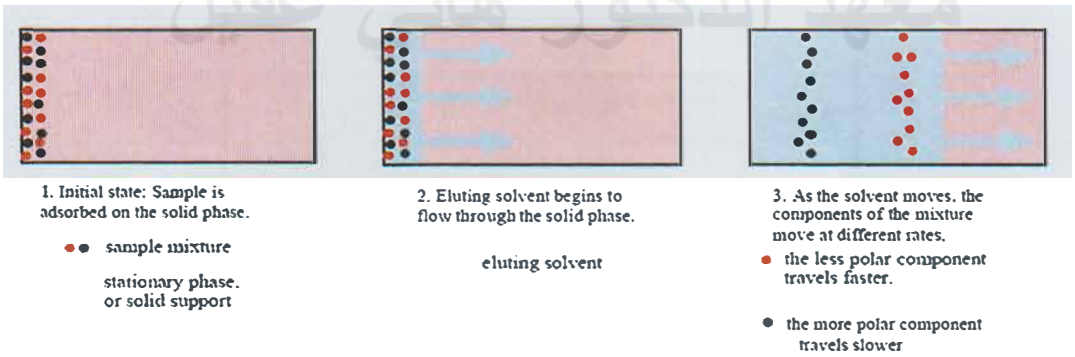


Figure 3: The separation principle of TLC.

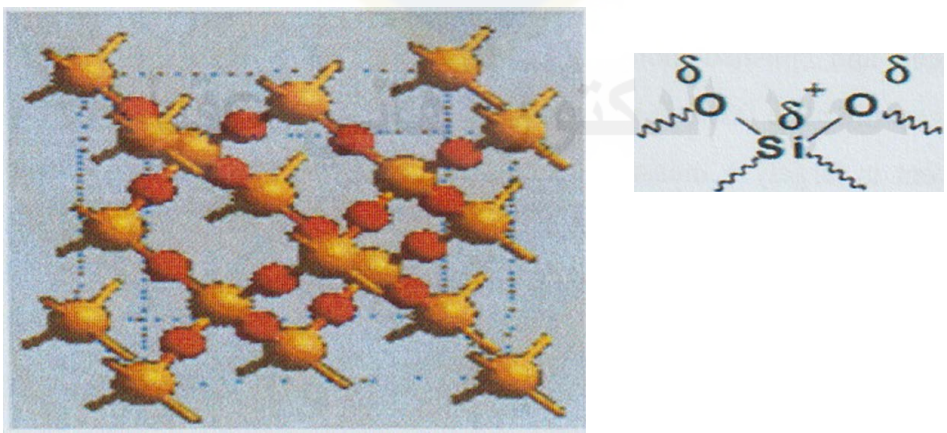
1.5.2. The components of TLC

❖ *Stationary phase*

In TLC, the stationary phase is typically alumina (Al_2O_3. xH_2O) n or silica gel (SiO_2. xH_2O) n. The covalent networks of these absorbents create very polar materials. The structure of silica is shown in picture.

The electropositive character of the aluminum or silicon and the electronegative oxygen create a very polar stationary phase. Therefore, the more polar the molecule to be separated, the stronger the attractive force to the stationary phase. The equilibrium will be shifted as the molecules remain on the stationary phase. Nonpolar molecules will have a lower affinity for the stationary phase and will remain in the solvent longer. This is essentially how the partitioning separates the molecules. The equilibrium governs the separation, but the component's attraction to the stationary phase versus the mobile phase determines the equilibrium.

Although alumina and silica are the most common stationary phases used for TLC, there are many different types. They range from paper to charcoal, nonpolar to polar, and reverse phase to normal phase. Several different types of stationary phases are listed according to polarity in figure below



Structure of Silica $(SiO_2 \cdot xH_2O)_n$

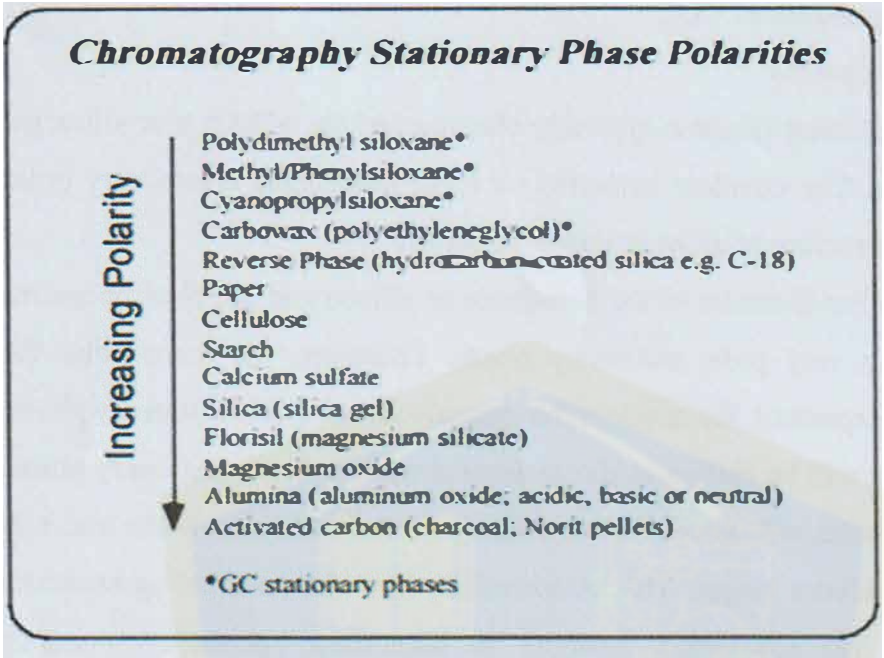


Figure 4: Common stationary phases listed by increasing polarity.

❖ Eluting phase

The most common factor that is adjusted to achieve good separation is the solvents used in the mobile phase. There are many choices for solvents and solvent mixtures are quiet common. The substances being separated are adsorbed onto the stationary phase, but polar solvent molecules are also adsorbed by the stationary phase. Molecules that are already adsorbed are displaced and "pushed along" by polar solvent molecules. Thus, everything moves up the plate faster in more polar solvent systems. The "Eluting power" of a solvent is largely a measure of how well it is adsorbed onto the stationary phase, displacing other molecules.

Eluting solvents for chromatography are shown in figure 5.

The non-polar solvents are often used as a base and a few percent of a stronger eluting, more polar solvent is added. As the eluting power of the added solvent increases, the amount that is generally added decreases. "Medium polar" solvents like diethyl ether and ethyl acetate may be used 1%-50% combination with hexane making these mixtures very tunable and common. Stronger eluting solvents like methanol cannot be used as more than 10% of the solution or the silica gel will dissolve in them causing problems with the separation. More than 1% of pyridine or acetic acid isn't often necessary, a drop or two is more common, while these two additives are next to each other on the list, and they can have very different effects on a separation depending on the functional groups in the molecules being separated. Water is very strongly eluting and its presence as an impurity in your solvent can be problematic. The functional groups of the molecules in your mixture effect how strongly they are adsorbed by the stationary phase. Very "greasy" non-polar substructures, usually made entirely of carbon and hydrogen, are hardly adsorbed by silica gel at all. Polar groups, with oxygen and especially nitrogen are more strongly adsorbed. The ability to hydrogen bond with the silica gel creates a strong adsorbing interaction in alcohols, carboxylic acids and amines.

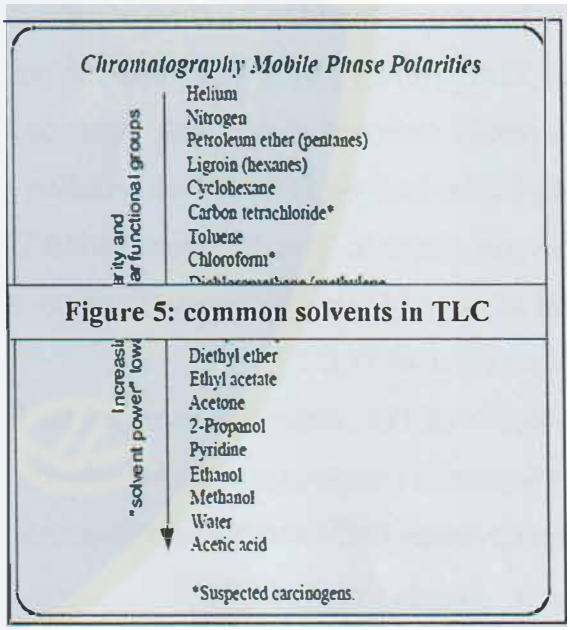


Figure 5: common solvents in TLC

1.5.3. Advantages and Disadvantages of TLC

- Advantages:

•Microscale techniques: only few milligrams of extracts are enough to run on TLC plate for analysis and identification.

• Rapid identifier: Sample identification in short time.

• Easy to monitor: a chromatography separation reaction.

• Easy determination: Number of compounds in a mixture could be easily determined.

• Bioautography: Identification of antimicrobial compounds can be done readily of TLC profiled plates. Here pouring potential test microbes (E.coli and Staph. aureous) cultures along with agar on resolved TLC plates can yield the inhibition band on TLC this will confirm an antibacterial activity

•Spray reagents: The developed, dried TLC plates are used for spraying. 5 – 10 ml solution of spray is sprayed from 10- 15 cm distance in the even manner over the surface of TLC .

•Resolved TLC plates can be stored for long duration time .

•Separated compounds may be subjected to Infrared Spectroscopy (IR), Mass spectrometry (MS) and Nuclear magnetic resonance (NMR).

- Disadvantages of TLC:

TLC plates do not have long stationary phases. Therefore, the length of separation is limited compared to other chromatographic techniques. Also, the detection limit is a lot higher. If would need a lower detection limit, one would have to use other chromatographic techniques. TLC operates as an open system, so factors such as humidity and temperature can be affect the results of the chromatogram.

1.5.4. Steps to run thin layer chromatography

- **Spotting of TLC plate**

One advantage TLC has over other separation methods is that it is truly a micro-scale technique. Only a few micrograms of material in solution are necessary to observe the solute on a TLC plate.

Dissolve a few milligrams of material in a volatile solvent creating a dilute solution. Choose a volatile solvent that completely dissolves the sample. However, if it is partially soluble, since such only low concentrations are needed, normally you will be able to observe the compound.

Once the sample is prepared, a spotting capillary must be used to add the sample to the plate. The spotting capillaries must be extremely small.

- **Development**

Once the dilute solution of the mixture has been spotted on the plate, the next step is the development. Just like paper chromatography, the solvent must be in contact with the stationary phase. The bottle is filled with a small amount of the mobile phase and capped with a cork. In addition, a piece of filter paper is put in the bottle to help create an atmosphere saturated with solvent. The origin spots are not below the solvent level in the chamber. If the spots are submerged in the solvent, they are washed off the plate and lost. Once the solvent has run within a centimeter of the top of the plate, remove it with tweezers.

Using a pencil, immediately draw a line across the plate where the solvent front can be seen. The proper location of this solvent front line will be important for later calculations.

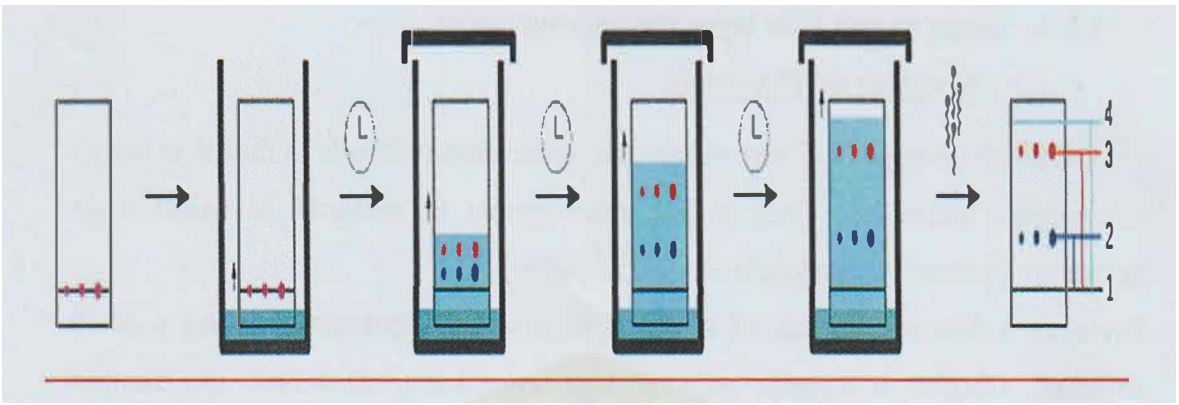


Figure 6: Application of spots and development of a TLC plate.

- **Visualization of TLC plate**

If components of the reaction are colored, no visualization method is required (*spots can be seen directly on the silica layer*). However, since most organic compounds are colorless; therefore one of the methods described below should be used to reveal the spots.

Non-destructive methods

As a general visualization procedure, TLC plates normally contain a fluorescent indicator which makes the TLC plate glow green under UV light of Wavelength 254 nm. Compounds that absorb UV light will quench the green fluorescence yielding dark purple or bluish spots on the plate (*usually for polyconjugated compounds like benzophenones and anthracenes*). Simply put the plate under a UV lamp, and the compounds become visible to the naked eye. Lightly circle the spots, so that you will have a permanent record.

Another useful visualizing technique is an iodine (I_2) chamber. Iodine sublimes and will absorb to organic molecules in the vapor phase. The organic spots on the plate will turn brown and can be easily identified. Also circle these observed spots, since the color stain will eventually fade from the plate.